

## Monoclonal Antibody 806 Inhibits the Growth of Tumor Xenografts Expressing Either the de2-7 or Amplified Epidermal Growth Factor Receptor (EGFR) but not Wild-Type EGFR

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### Abstract

The monoclonal antibody (mAb) 806 was raised against the delta2-7 epidermal growth factor receptor (de2-7 EGFR or EGFRvIII), a truncated version of the EGFR commonly expressed in glioma. Unexpectedly, mAb 806 also bound the EGFR expressed by cells exhibiting amplification of the *EGFR* gene but not to cells or normal tissue expressing the wild-type receptor in the absence of gene amplification. The unique specificity of mAb 806 offers an advantage over current EGFR antibodies, which all display significant binding to the liver and skin in humans. Therefore, we examined the antitumor activity of mAb 806 against human tumor xenografts grown in nude mice. The growth of U87 MG xenografts, a glioma cell line that endogenously expresses ~10<sup>5</sup> EGFRs in the absence of gene amplification, was not inhibited by mAb 806. In contrast, mAb 806 significantly inhibited the growth of U87 MG xenografts transfected with the de2-7 EGFR in a dose-dependent manner using both preventative and established tumor models. Significantly, U87 MG cells transfected with the wild-type EGFR, which increased expression to ~10<sup>6</sup> EGFRs/cell and mimics the situation of gene amplification, were also inhibited by mAb 806 when grown as xenografts in nude mice. Xenografts treated with mAb 806 all displayed large areas of necrosis that were absent in control tumors. This reduced xenograft viability was not mediated by receptor down-regulation or clonal selection because levels of antigen expression were similar in control and treated groups. The antitumor effect of mAb 806 was not restricted to U87 MG cells because the antibody inhibited the growth of new and established A431 xenografts, a cell line expressing >10<sup>6</sup> EGFRs/cell. This study demonstrates that mAb 806 possesses significant antitumor activity.

### Introduction

Over the past two decades, mAbs<sup>2</sup> have attracted considerable interest as potential agents for the treatment of human cancer (1, 2). A number of these mAbs have been directed to the EGFR, which given its increased expression on the cell surface of many human tumors is a candidate for antibody therapy. Overexpression of the EGFR has been observed in tumors of the breast, lung, colon, prostate, kidney, bladder, head and neck, ovary, and brain (3, 4), with increased EGFR expression levels often correlating with a poorer clinical prognosis (5, 6). Overexpression of the EGFR can be associated with *EGFR* gene amplification, particularly in glioma and head and neck cancer.

Some of the current EGFR-specific mAbs are capable of inhibiting the *in vitro* and *in vivo* growth of epithelial tumor cells overexpressing the EGFR by blocking ligand binding (7-10). Although several EGFR mAbs have been evaluated for tumor-specific targeting and pharmacokinetics in clinical trials (11, 12), their use is restricted by specific uptake in organs that have high endogenous levels of EGFR, such as the liver and skin (11, 13). For example, it would not be possible to conjugate such antibodies to cytotoxic agents for the purposes of therapy because this would almost certainly cause significant collateral damage to normal tissue.

*EGFR* gene amplification in glioma is often accompanied by gene rearrangement, resulting in deletions of the coding region (14). The most common variant, the de2-7 EGFR, is characterized by an in-frame deletion of 801 bp spanning exons 2-7 of the coding sequence (15). This truncation removes 267 amino acids from the extracellular domain, producing a unique junctional peptide, and renders the EGFR unable to bind any known ligand (4). Despite this, the truncation appears to partially mimic the effect of ligand binding because the receptor displays low levels of constitutive activation (16). Furthermore, glioma and breast cells transfected with the de2-7 EGFR have enhanced tumorigenicity when grown as xenografts in nude mice (16, 17). Apart from glioma, the de2-7 EGFR has been identified in breast, non-small cell lung, ovarian, and prostate cancer (3, 18, 19) but has not been found in normal tissue (20). Therefore, targeting this tumor-specific antigen may permit broader therapeutic strategies than is possible using wt EGFR-based immunotherapeutic strategies. Indeed, several mAbs specific for the unique junctional peptide found in the de2-7 EGFR have been described (20-22).

The de2-7 EGFR specific mAb 806 was produced after immunization of mice with NR6 mouse fibroblasts expressing the truncated de2-7 EGFR. mAb 806 binds the U87 MG glioma cell line transfected with the de2-7 EGFR but not the parental U87 MG cell line, which expresses the wt EGFR without gene amplification.<sup>3</sup> Similar results were observed *in vivo* with mAb 806 showing specific targeting of de2-7 EGFR expressing U87 MG xenografts but not parental U87 MG tumors.<sup>3</sup> Interestingly, mAb 806 was capable of binding an EGFR subset (~10%) on the surface of the A431 cell line, which contains an amplified *EGFR* gene. Therefore, unlike all other de2-7 EGFR-specific antibodies, which recognize the unique peptide junction that is generated by the de2-7 EGFR truncation, mAb 806 binds to an epitope also found in overexpressed wt EGFR. However, it

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<sup>2</sup> The abbreviations used are: mAb, monoclonal antibody; de2-7, delta2-7; EGFR, epidermal growth factor receptor; wt, wild type; FCS, fluorescence-activated cell sorter; HSA, human serum albumin.

<sup>3</sup> T. G. Johns, E. Stockert, G. Ritter, A. A. Jungbluth, H-J. S. Huang, W. K. Cavenee, F. E. Smyth, C. M. Hall, N. Watson, E. C. Nice, L. J. Old, A. W. Burgess, and A. M. Scott. A novel monoclonal antibody specific for the de2-7 epidermal growth factor receptor (EGFR) that also recognizes the EGFR expressed in cells containing amplification of the *EGFR* gene, submitted for publication.

would appear that this epitope is preferentially exposed in the de2-7 EGFR and a small proportion of receptors expressed in cells containing wt *EGFR* gene amplification. Importantly, normal tissues that expresses high levels of endogenous wt EGFR, such as liver and skin, are negative for mAb 806 binding. On the basis of the unique property of the mAb 806 to bind both the de2-7 and amplified wt EGFR but not the native wt EGFR when expressed at normal levels, we decided to examine the efficacy of mAb 806 against several tumor cell lines grown as xenografts in nude mice.

## Materials and Methods

**Cell Lines and Monoclonal Antibodies.** The human glioblastoma cell line U87 MG, which endogenously expresses the wt EGFR, and the transfected cell lines U87 MG.Δ2-7 and U87 MG.wtEGFR, which express the de2-7 EGFR and overexpress the wt EGFR, respectively, have been described previously (16, 23). The epidermoid carcinoma cell line A431 has been described previously (24).

All cell lines were maintained in DMEM (DMEM/F12; Life Technologies, Inc., Grand Island, NY) containing 10% FCS (CSL, Melbourne, Victoria, Australia), 2 mM glutamine (Sigma Chemical Co., St. Louis, MO), and penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY). In addition, the U87 MG.Δ2-7 and U87 MG.wtEGFR cell lines were maintained in 400 μg/ml of geneticin (Life Technologies, Inc., Melbourne, Victoria, Australia). Cell lines were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

The mAb 806 (IgG2b) was produced after immunization of mice with NR6 mouse fibroblasts expressing the de2-7 EGFR. mAb 806 was selected after rosette assays showed binding to NR6 cells, which overexpressed the de2-7 EGFR (titer of 1:2500). mAb 528, which recognizes both de2-7 and wt EGFR, has been described previously (10) and was produced in the Biological Production Facility (Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia) using a hybridoma obtained from American Type Culture Collection (Rockville, MD). The DH8.3 mAb, which is specific for the de2-7 EGFR, was kindly provided by Prof. William Gullick (University of Kent and Canterbury, Kent, United Kingdom) (19). The polyclonal antibody sc-03 directed to the COOH-terminal domain of the EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA).

**FACS Analysis of Receptor Expression.** Cultured parental and transfected U87 MG cell lines were analyzed for wt and de2-7 EGFR expression using the 528, 806, and DH8.3 antibodies. Cells ( $1 \times 10^6$ ) were incubated with 5 μg/ml of the appropriate antibody or an isotype-matched negative control in PBS containing 1% HSA for 30 min at 4°C. After three washes with PBS/1% HSA, cells were incubated an additional 30 min at 4°C with FITC-coupled goat antimouse antibody (1:100 dilution; Calbiochem, San Diego, CA). After three subsequent washes, cells were analyzed on an Epics Elite ESP (Beckman Coulter, Hialeah, FL) by observing a minimum of 20,000 events and analyzed using EXPO (version 2) for Windows.

**Scatchard Analysis.** The mAb 806 was labeled with <sup>125</sup>I (Amrad, Melbourne, Victoria, Australia) by the Chloramine T method. All binding assays were performed in 1% HSA/PBS on  $1-2 \times 10^6$  live U87 MG.Δ2-7 or A431 cells for 90 min at 4°C with gentle rotation. A set concentration of 10 ng/ml <sup>125</sup>I-labeled mAb 806 was used in the presence of increasing concentrations of unlabeled antibody. Nonspecific binding was determined in the presence of 10,000-fold excess of unlabeled antibody. After incubation, cells were washed and counted for bound <sup>125</sup>I-labeled mAb 806 using a COBRA II gamma counter (Packard Instrument Company, Meriden, CT). Scatchard analysis was done after correction for immunoreactivity.

**Immunoprecipitation Studies.** Cells were labeled for 16 h with 100 μCi/ml of Tran<sup>35</sup>S-Label (ICN Biomedicals, Irvine, CA) in DMEM without methionine/cysteine supplemented with 5% dialyzed FCS. After washing with PBS, cells were placed in lysis buffer (1% Triton X-100, 30 mM HEPES, 150 mM NaCl, 500 μM 4-(2-aminoethyl) benzenesulfonyl fluoride, 150 mM aprotinin, 1 μM E-64 protease inhibitor, 0.5 mM EDTA, and 1 μM leupeptin, pH 7.4) for 1 h at 4°C. Lysates were clarified by centrifugation for 10 min at 12,000 × g and then incubated with 5 μg of appropriate antibody for 30 min at 4°C before the addition of protein A-Sepharose. Immunoprecipitates were washed three times with lysis buffer, mixed with SDS sample buffer, separated

by gel electrophoresis using a 7.5% gel that was then dried, and exposed to X-ray film.

**Xenograft Models.** Consistent with previous reports (23, 25), U87 MG cells transfected with de2-7 EGFR grew more rapidly than parental cells and U87 MG cells transfected with the wt EGFR. Tumor cells ( $3 \times 10^6$ ) in 100 μl of PBS were inoculated s.c. into both flanks of 4-6-week-old, female nude mice (Animal Research Center, Western Australia, Perth, Australia). Therapeutic efficacy of mAb 806 was investigated in both preventative and established tumor models. In the preventative model, five mice with two xenografts each were treated i.p. with either 0.1 or 1 mg of mAb 806 or vehicle (PBS) starting the day before tumor cell inoculation. Treatment was continued for a total of six doses, three times per week for 2 weeks. In the established model, treatment was started when tumors had reached a mean volume of 65 mm<sup>3</sup> (U87 MG.Δ2-7), 84 mm<sup>3</sup> (U87 MG), 73 mm<sup>3</sup> (U87 MG.wtEGFR), or 201 mm<sup>3</sup> (A431 tumors). Tumor volume in mm<sup>3</sup> was determined using the formula (length × width<sup>2</sup>)/2, where length was the longest axis and width the measurement at right angles to the length (26). Data were expressed as mean tumor volume ± SE for each treatment group. This research project was approved by the Animal Ethics Committee of the Austin and Repatriation Medical Centre.

**Histological Examination of Tumor Xenografts.** Xenografts were excised at the times indicated and bisected. One half was fixed in 10% formalin/PBS before being embedded in paraffin. Four-μm sections were then cut and stained with H&E for routine histological examination. The other half was embedded in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and stored at -80°C. Thin (5-μm) cryostat sections were cut and fixed in ice-cold acetone for 10 min, followed by air drying for an additional 10 min. Sections were blocked in protein blocking reagent (Lipshaw Immunon, Pittsburgh, PA) for 10 min and then incubated with biotinylated primary antibody (1 μg/ml) for 30 min at room temperature. All antibodies were biotinylated using the ECL protein biotinylation module (Amersham, Baulkham Hills, NSW, Australia), as per the manufacturer's instructions. After rinsing with PBS, sections were incubated with a streptavidin-horseradish peroxidase complex for an additional 30 min (Silenus, Melbourne, Victoria, Australia). After a final PBS wash, the sections were exposed to 3-amino-9-ethylcarbazole substrate [0.1 M acetic acid, 0.1 M sodium acetate, 0.02 M 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO)] in the presence of hydrogen peroxide for 30 min. Sections were rinsed with water and counterstained with hematoxylin for 5 min and mounted.

**Statistical Analysis.** The *in vivo* tumor measurements in mm<sup>3</sup> are expressed as the mean ± SE. Differences between treatment groups at given time points were tested for statistical significance using Student's *t* test.

## Results

**Binding of Antibodies to Cell Lines.** To determine the specificity of mAb 806, its binding to U87 MG, U87 MG.Δ2-7, and U87 MG.wtEGFR cells was analyzed by FACS. An irrelevant IgG2b (mAb 100-310 directed to the human antigen A33) was included as an isotype control for mAb 806, and the 528 antibody was included because it recognizes both the de2-7 and wt EGFR. Only the 528 antibody was able to stain the parental U87 MG cell line (Fig. 1), consistent with previous reports demonstrating that these cells express the wt EGFR (16). mAb 806 had binding levels similar to the control antibody, clearly demonstrating that it is unable to bind the wt EGFR (Fig. 1). Binding of the isotype control antibody to the U87 MG.Δ2-7 and U87 MG.wtEGFR cell lines was similar to that observed for the U87 MG cells. mAb 806 stained U87 MG.Δ2-7 and U87 MG.wtEGFR cells, indicating that mAb 806 specifically recognized the de2-7 EGFR and a subset of the overexpressed EGFR (Fig. 1). As expected, the 528 antibody stained both the U87 MG.Δ2-7 and U87 MG.wtEGFR cell lines (Fig. 1). The intensity of 528 antibody staining on U87 MG.wtEGFR cells was much higher than mAb 806, suggesting that mAb 806 only recognizes a portion of the overexpressed EGFR. The mAb 806 reactivity observed with U87 MG.wtEGFR cells is similar to that obtained with A431 cells, another cell line that overexpresses the wt EGFR.<sup>3</sup>

A Scatchard analysis was performed using U87 MG.Δ2-7 and

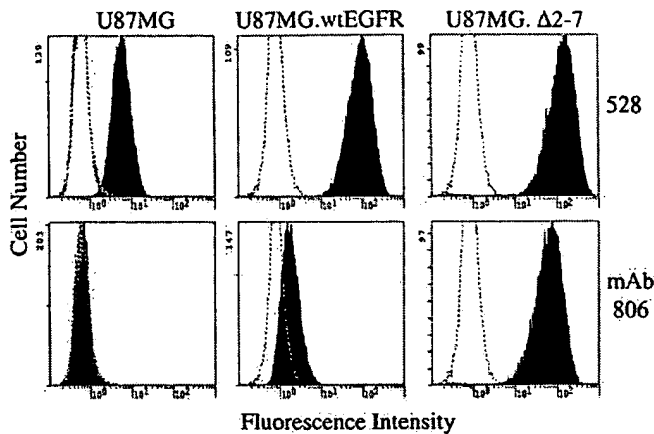


Fig. 1. Flow cytometric analysis of parental and transfected U87 MG glioma cell lines. Cells were stained with either an irrelevant IgG2b antibody (open histograms) or the 528 antibody or mAb 806 (filled histograms) as indicated.

A431 cells to determine the relative affinity and binding sites for mAb 806 on each cell line. mAb 806 had an affinity for the de2-7 EGFR receptor of  $1.1 \times 10^9 \text{ M}^{-1}$  and recognized an average (three separate experiments) of  $2.4 \times 10^5$  binding sites/cell. In contrast, the affinity of mAb 806 for the wt EGFR on A431 cells was only  $9.5 \times 10^7 \text{ M}^{-1}$ . Interestingly, mAb 806 recognized  $2.3 \times 10^5$  binding sites on the surface of A431, which is some 10-fold lower than the reported number of EGFR found in these cells. To confirm the number of EGFR on the surface of our A431 cells, we performed a Scatchard analysis using  $^{125}\text{I}$ -labeled 528 antibody. As expected, this antibody bound to approximately  $2 \times 10^6$  sites on the surface of A431 cells. Thus, it appears that mAb 806 only binds a portion of the EGFR receptors on the surface of A431 cells. Importantly,  $^{125}\text{I}$ -labeled mAb 806 did not bind to the parental U87 MG cells at all, even when the number of cells was increased to  $1 \times 10^7$ .

**Immunoprecipitations.** We further characterized mAb 806 reactivity in the various cell lines by immunoprecipitation after  $^{35}\text{S}$ -labeling using mAb 806, sc-03 (a commercial polyclonal antibody specific for the COOH-terminal domain of the EGFR) and a IgG2b isotype control. The sc-03 antibody immunoprecipitated three bands from U87 MG.Δ2-7 cells, a doublet corresponding to the two de2-7 EGFR bands observed in these cells and a higher molecular weight band corresponding to the wt EGFR (Fig. 2). In contrast, although mAb 806 immunoprecipitated the two de2-7 EGFR bands, the wt EGFR was completely absent (Fig. 2). The pattern seen in U87 MG.wtEGFR and A431 cells was essentially identical. The sc-03 antibody immunoprecipitated a single band corresponding to the wt EGFR from both cell lines (Fig. 2). The mAb 806 also immunoprecipitated a single band corresponding to the wt EGFR from both U87 MG.wtEGFR and A431 cells (Fig. 2). Consistent with the FACS and Scatchard data, the amount of EGFR immunoprecipitated by mAb 806 was substantially less than the total EGFR present on the cell surface. Given that mAb 806 and the sc-03 immunoprecipitated similar amounts of the de2-7 EGFR, this result supports the notion that the mAb 806 antibody only recognizes a portion of the EGFR in cells overexpressing the receptor. Comparisons between mAb 806 and the 528 antibody showed an identical pattern of reactivity (data not shown). An irrelevant IgG2b (an isotype control for mAb 806) did not immunoprecipitate EGFR from any of the cell lines (Fig. 2). Using identical conditions, mAb 806 did not immunoprecipitate the EGFR from the parental U87 MG cells (data not shown).

**Efficacy of mAb 806 in Preventative Models.** mAb 806 was examined for efficacy against U87 MG and U87 MG.Δ2-7 tumors in

a preventative xenograft model. Antibody or vehicle was administered i.p. the day before tumor inoculation and was given three times per week for 2 weeks (see "Materials and Methods"). At a dose of 1 mg/injection, mAb 806 had no effect on the growth of parental U87 MG xenografts that express the wt EGFR (Fig. 3A). In contrast, mAb 806 inhibited significantly the growth of U87 MG.Δ2-7 xenografts in a dose-dependent manner (Fig. 3B). Twenty days after tumor inoculation, when control animals were sacrificed, the mean tumor volume was  $1600 \pm 180 \text{ mm}^3$  for the control group, a significantly smaller  $500 \pm 95 \text{ mm}^3$  for the 0.1 mg/injection group ( $P < 0.0001$ ) and  $200 \pm 42 \text{ mm}^3$  for the 1 mg/injection group ( $P < 0.0001$ ). Treatment groups were sacrificed at day 24, at which time the mean tumor volumes were  $1300 \pm 240 \text{ mm}^3$  for the 0.1 mg treated group and  $500 \pm 100 \text{ mm}^3$  for the 1 mg group ( $P < 0.005$ ).

**Efficacy of mAb 806 in Established Xenograft Models.** Given the efficacy of mAb 806 in the preventative xenograft model, its

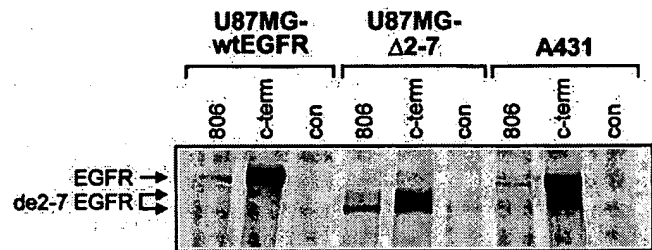


Fig. 2. Immunoprecipitation of EGFR from cell lines. The EGFR was immunoprecipitated from  $^{35}\text{S}$ -labeled U87 MG.wtEGFR, U87 MG.Δ2-7, and A431 cells with mAb 806 (806), sc-03 antibody (c-term), or a IgG2b isotype control (con) as described in "Materials and Methods." Arrows, position of the de2-7 and wt EGFR. Identical banding patterns were obtained in three independent experiments.

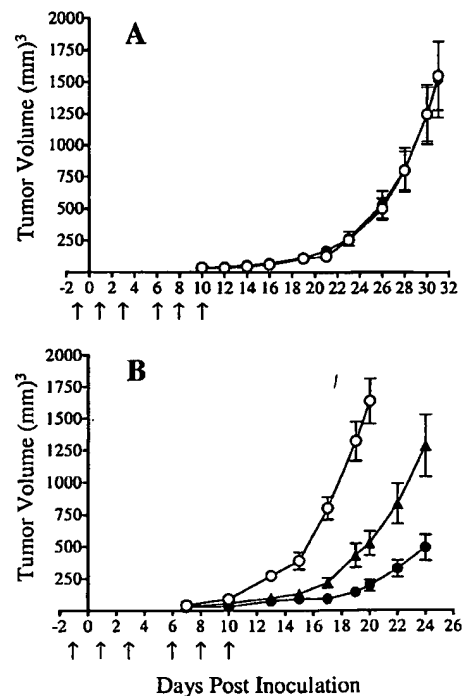


Fig. 3. Antitumor effect of mAb 806 on U87 MG (A) and U87 MG.Δ2-7 (B) xenograft growth rates in a preventative model. U87 MG or U87 MG.Δ2-7 cells ( $3 \times 10^6$ ) were injected s.c. into both flanks of 4–6-week-old BALB/c nude mice ( $n = 5$ ) at day 0. Mice were injected i.p. with either 1 mg of mAb 806 (●), 0.1 mg of mAb 806 (▲), or vehicle (○) starting 1 day prior to tumor cell inoculation. Injections of mAb 806 were given three times/week for 2 weeks (arrows). Data are expressed as mean tumor volume; bars, SE.

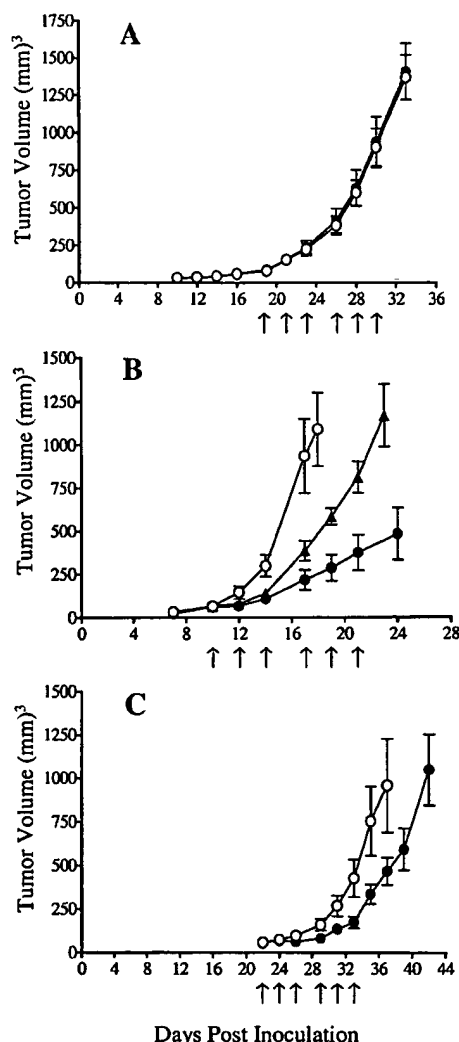


Fig. 4. Antitumor effect of mAb 806 on U87 MG (A), U87 MG.Δ2-7 (B), and U87 MG.wtEGFR (C) xenografts in an established model. U87 MG, U87 MG.Δ2-7, or U87 MG.wtEGFR cells ( $3 \times 10^6$ ) were injected s.c. into both flanks of 4–6-week-old BALB/c nude mice ( $n = 5$ ). Mice were injected i.p. with either 1-mg doses of mAb 806 (●), 0.1-mg doses of mAb 806 (▲), or vehicle (○) starting when tumors had reached a mean tumor volume of 65–84 mm<sup>3</sup>. Injections were given three times/week for 2 weeks (arrows). Data are expressed as mean tumor volume; bars, SE.

ability to inhibit the growth of established tumor xenografts was examined. Antibody treatment was as described in the preventative model, except that it commenced when tumors had reached a mean tumor volume of 65 mm<sup>3</sup> (10 days after implantation) for the U87 MG.Δ2-7 xenografts and 84 mm<sup>3</sup> (19 days after implantation) for the parental U87 MG xenografts. Once again, mAb 806 had no effect on the growth of parental U87 MG xenografts, even at a dose of 1 mg/injection (Fig. 4A). In contrast, mAb 806 significantly inhibited the growth of U87 MG.Δ2-7 xenografts in a dose-dependent manner (Fig. 4B). At day 17, 1 day before control animals were sacrificed, the mean tumor volume was  $900 \pm 200$  mm<sup>3</sup> for the control group,  $400 \pm 60$  mm<sup>3</sup> for the 0.1 mg/injection group ( $P < 0.01$ ), and  $220 \pm 60$  mm<sup>3</sup> for the 1 mg/injection group ( $P < 0.002$ ). Treatment of U87 MG.Δ2-7 xenografts with an IgG2b isotype control had no effect on tumor growth (data not shown).

To examine whether the growth inhibition observed with mAb 806 was restricted to cells expressing de2-7 EGFR, its efficacy against the U87 MG.wtEGFR xenografts was also examined in an established

model. These cells serve as a model for tumors containing amplification of the *EGFR* gene without de2-7 EGFR expression. mAb 806 treatment commenced when tumors had reached a mean tumor volume of 73 mm<sup>3</sup> (22 days after implantation). mAb 806 significantly inhibited the growth of established U87 MG.wtEGFR xenografts when compared with control tumors treated with vehicle (Fig. 4C). On the day control animals were sacrificed, the mean tumor volume was  $1000 \pm 300$  mm<sup>3</sup> for the control group and  $500 \pm 80$  mm<sup>3</sup> for the group treated with 1 mg/injection ( $P < 0.04$ ).

**Histological and Immunohistochemical Analysis of Established Tumors.** To evaluate potential histological differences between mAb 806-treated and control U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts, formalin-fixed, paraffin-embedded sections were stained with H&E (Fig. 5). Areas of necrosis were seen in sections from mAb 806-treated U87 MG.Δ2-7 (mAb 806-treated xenografts were collected 24 days after tumor inoculation and vehicle treated xenografts at 18 days), and U87 MG.wtEGFR xenografts (mAb 806 xenografts were collected 42 days after tumor inoculation and vehicle treated xenografts at 37 days; Fig. 5). This result was consistently observed in a number of tumor xenografts ( $n = 4$  for each cell line). However, sections from U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts treated with vehicle ( $n = 5$ ) did not display the same areas of necrosis seen after mAb 806 treatment (Fig. 5). Vehicle and mAb 806-treated xenografts removed at identical times also showed these differences in tumor necrosis (data not shown). Thus, the increase in necrosis observed was not caused by the longer growth periods used for the mAb 806-treated xenografts. Furthermore, sections from mAb 806-treated U87 MG xenografts were also stained with H&E and did not reveal any areas of necrosis (data not shown), further supporting the hypothesis that mAb 806 binding induces decreased cell viability, resulting in increased necrosis within tumor xenografts.

An immunohistochemical analysis of U87 MG, U87 MG.Δ2-7, and U87 MG.wtEGFR xenograft sections was performed to determine the levels of de2-7 and wt EGFR expression after mAb 806 treatment (Fig. 6). As expected, the 528 antibody stained all xenografts sections with no obvious decrease in intensity between treated and control tumors (Fig. 6). Staining of U87 MG sections was undetectable with the mAb 806; however, positive staining of U87 MG.Δ2-7 and U87 MG.wtEGFR xenograft sections was observed (Fig. 6). There was no

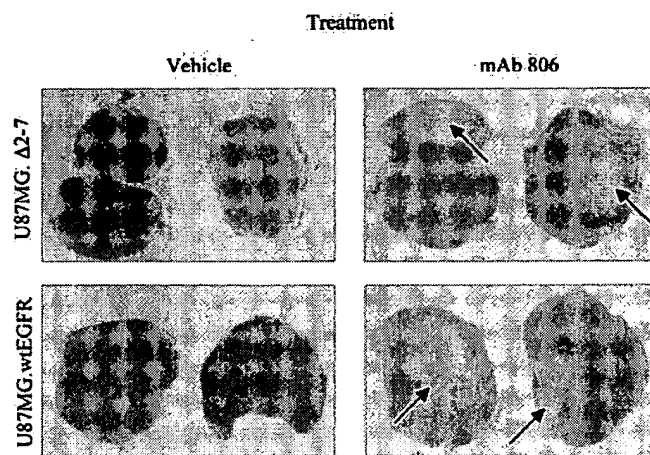


Fig. 5. Representative H&E-stained paraffin sections of U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts. U87 MG.Δ2-7 (collected 24 days after tumor inoculation) and U87 MG.wtEGFR (collected 42 days after tumor inoculation) xenografts were excised from mice treated as described in Fig. 4 and stained with H&E. Vehicle-treated U87 MG.Δ2-7 (collected 18 days after tumor inoculation) and U87 MG.wtEGFR (collected 37 days after tumor inoculation) xenografts showed very few areas of necrosis (left panel), whereas extensive necrosis (arrows) was observed in both U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts treated with mAb 806 (right panel).

## Stained for 528 reactivity

## Stained for mAb 806 reactivity

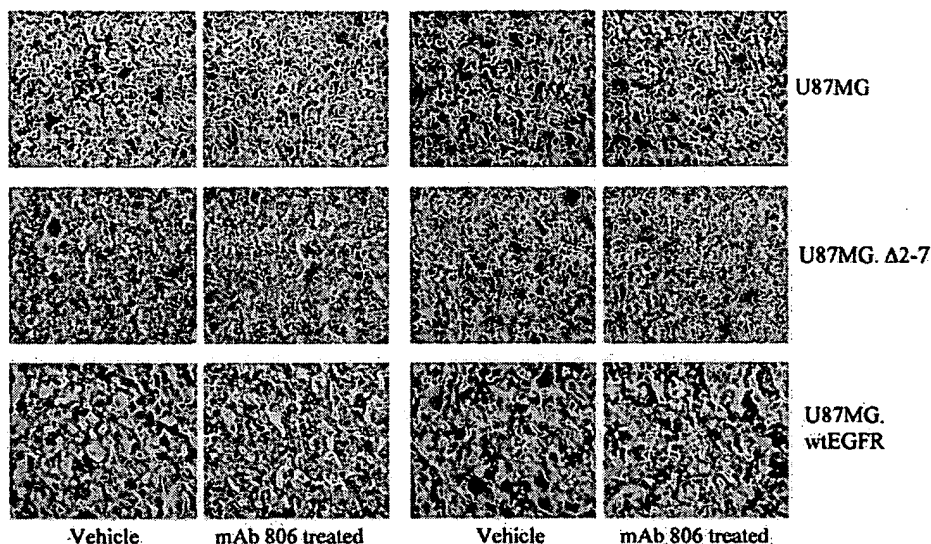


Fig. 6. Immunohistochemical analysis of EGFR expression in frozen sections derived from U87 MG, U87 MG.Δ2-7, and U87 MG.wtEGFR xenografts. Sections were collected at the time points described in Fig. 5. Xenograft sections were immunostained with the 528 antibody (left panel) and mAb 806 (right panel). No decreased immunoreactivity to either wt EGFR, amplified EGFR, or de2-7 EGFR was observed in xenografts treated with mAb 806. Consistent with the *in vitro* data, parental U87 MG xenografts were positive for 528 antibody but were negative for mAb 806 staining.

difference in mAb 806 staining intensity between control and treated U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts, suggesting that antibody treatment does not lead to the selection of clonal variants lacking mAb 806 reactivity.

**Treatment of A431 Xenografts with mAb 806.** To demonstrate that the antitumor effects of mAb 806 were not restricted to U87 MG cells, the antibody was administered to mice containing A431 xenografts. These cells contain an amplified *EGFR* gene and express approximately  $2 \times 10^6$  receptors/cells. We have previously shown that mAb 806 binds  $\sim 10\%$  of these EGFRs and targets A431 xenografts.<sup>3</sup> mAb 806 significantly inhibited the growth of A431 xenografts when examined in the preventative xenograft model described previously (Fig. 7A). At day 13, when control animals were sacrificed, the mean tumor volume was  $1400 \pm 150 \text{ mm}^3$  in the vehicle-treated group and  $260 \pm 60 \text{ mm}^3$  for the 1 mg/injection treatment group ( $P < 0.0001$ ). In a separate experiment, a dose of 0.1 mg of mAb also inhibited significantly ( $P < 0.05$ ) the growth of A431 xenografts in a preventative model (data not shown).

Given the efficacy of mAb 806 in the preventative A431 xenograft model, its ability to inhibit the growth of established tumor xenografts was examined. Antibody treatment was as described in the preventative model, except it was not started until tumors had reached a mean tumor volume of  $200 \pm 20 \text{ mm}^3$ . mAb 806 significantly inhibited the growth of established A431 xenografts (Fig. 7B). At day 13, the day control animals were sacrificed, the mean tumor volume was  $1100 \pm 100 \text{ mm}^3$  for the control group and  $450 \pm 70 \text{ mm}^3$  for the 1 mg/injection group ( $P < 0.0001$ ).

## Discussion

Many epithelial cancers display increased levels of EGFR expression on the cell surface, and numerous experiments with EGFR mAbs have shown that they inhibit tumor cell growth both *in vitro* and *in vivo* (7, 9, 10, 27). Furthermore, clinical studies using EGFR mAbs in patients with head and neck cancer (28), squamous cell lung cancer (12), gliomas (29), and malignant astrocytomas (30) have all been conducted. However, the clinical use of these antibodies is complicated by liver uptake, as reported in a Phase I clinical trial (12).

An alternative and more specific target for antibody therapy is the tumor-specific de2-7 EGFR, which unlike the wt EGFR, is absent in

normal tissue (4, 14). A number of mAbs have been raised to the unique junctional peptide found in the de2-7 EGFR; these mAbs do not recognize the wt receptor (20-22) and specifically target de2-7 EGFR-positive xenografts grown in nude mice (31, 32). The use of these antibodies should not be complicated by uptake in normal tissues, such as liver and skin. However, with the exception of

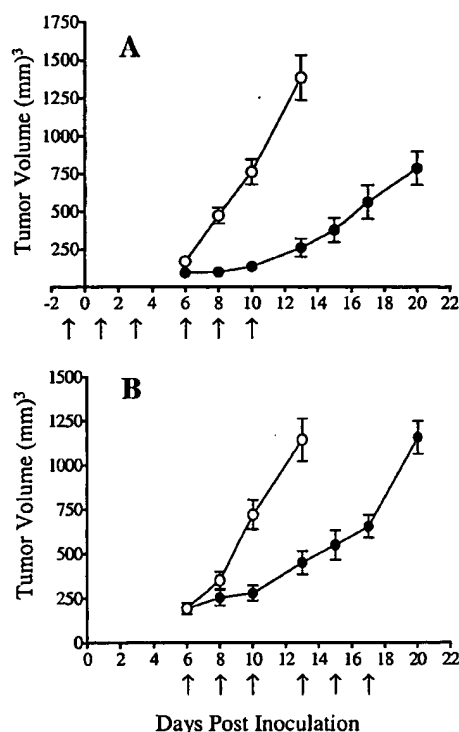


Fig. 7. Antitumor effect of mAb 806 on A431 xenografts in preventative (A) and established (B) models. A431 cells ( $3 \times 10^6$ ) were injected s.c. into both flanks of 4-6-week-old BALB/c nude mice ( $n = 5$ ). Mice were injected i.p. with either 1-mg doses of mAb 806 (●) or vehicle (○), starting 1 day prior to tumor cell inoculation in the preventative model, or when tumors had reached a mean tumor volume of  $200 \text{ mm}^3$ . Injections were given three times/week for 2 weeks (arrows). Data are expressed as mean tumor volume; bars, SE.

gliomas, only a small percentage of most carcinomas express the de2-7 EGFR, apparently restricting the use of such antibodies. We have shown previously<sup>3</sup> that mAb 806 targets both de2-7 EGFR-transfected U87 MG xenografts and A431 xenografts that overexpress the wt EGFR. mAb 806 did not target parental U87 MG cells, which express  $\sim 10^5$  EGFR<sup>3</sup> (16). As assessed by FACS, immunohistochemistry, and immunoprecipitation, we now demonstrate that mAb 806 is also able to specifically bind U87 MG.wtEGFR cells, which express  $>10^6$  EGFRs/cell. Thus, the previous observed binding of mAb 806 to A431 cells is not the result of some unusual property of these cells but rather appears to be a more general phenomenon related to overexpression of the wt EGFR.

Our data cannot completely exclude the possibility that mAb 806 binds to the wt EGFR with low affinity; however, for several reasons we favor the hypothesis that mAb 806 recognizes a subset of receptors in cells overexpressing the receptor:

(a) We were unable to detect mAb 806 binding to the parental U87 MG cell line, which expresses  $1 \times 10^5$  wt EGFRs/cell (16), either by FACS, immunoprecipitation, immunohistochemistry, or with iodinated antibody. Indeed, iodinated mAb 806 did not bind to U87 MG cell pellets containing  $1 \times 10^7$  cells, which based on the Scatchard data using  $1 \times 10^6$  A431 cells, are conditions that should detect low level antibody binding (*i.e.*, the total number of receptors being similar in both cases).

(b) Scatchard analysis clearly showed that mAb 806 only bound to 10% of the total EGFR on the surface of A431 cells. If mAb 806 simply binds to the wt EGFR with low affinity, then it should have bound to a considerably higher percentage of the receptor.

(c) Comparative immunoprecipitation of the A431 and U87 MG.wtEGFR cell lines with mAb 806 and the sc-03 antibody also supported the hypothesis that only a subset of receptors are recognized by mAb 806. Taken together, these results support the notion that mAb 806 recognizes a EGFR subset on the surface of cells overexpressing the EGFR. We are currently analyzing the EGFR immunoprecipitated by mAb 806 to see if it displays altered biochemical properties related to glycosylation or kinase activity.

The xenograft studies with mAb 806 described here demonstrate dose-dependent inhibition of U87 MG. $\Delta$ 2-7 xenograft growth. In contrast, no inhibition of parental U87 MG xenografts was observed, despite the fact that they continue to express the wt EGFR *in vivo*. mAb 806 not only significantly reduced xenograft volume, it also induced significant necrosis within the tumor. As noted above, other de2-7 EGFR-specific mAbs have been generated (20-22), but this is the first report showing the successful therapeutic use of such an antibody *in vivo* against a human de2-7 EGFR-expressing glioma xenograft. A recent report demonstrated that the de2-7 EGFR-specific Y10 mAb had *in vivo* antitumor activity against murine B16 melanoma cells transfected with a murine homologue of the human de2-7 EGFR (33). Y10 mediated *in vitro* cell lysis ( $>90\%$ ) of B16 melanoma cells expressing the de2-7 EGFR in the absence of complement or effector cells. In contrast to their *in vitro* observations, the *in vivo* Y10 antibody efficacy was completely mediated through Fc function when using B16 melanoma cells grown as xenografts in an immunocompetent model. Thus, the direct effects observed *in vitro* do not seem to be replicated when cells are grown as tumor xenografts.

Overexpression of the EGFR has been reported in a number of different tumors and is observed in most gliomas (4, 14). It has been proposed that the subsequent EGFR overexpression mediated by receptor gene amplification may confer a growth advantage by increasing intracellular signaling and cell growth (34). The U87 MG cell line was transfected with the wt EGFR to produce a glioma cell that mimics the process of EGFR gene amplification. Treatment of established U87 MG.wtEGFR xenografts with mAb 806 resulted in sig-

nificant growth inhibition. Thus, mAb 806 also mediates *in vivo* antitumor activity against cells overexpressing the EGFR. Interestingly, mAb 806 inhibition of U87 MG.wtEGFR xenografts was less pronounced than that observed with U87 MG. $\Delta$ 2-7 tumors. This probably reflects the fact that mAb 806 has a lower affinity for the overexpressed wt EGFR and only binds a small proportion of receptors expressed on the cell surface.<sup>3</sup> However, it should be noted that despite the small effect on U87 MG.wtEGFR xenograft volumes, mAb 806 treatment produced large areas of necrosis within these xenografts. To exclude the possibility that mAb 806 only mediates inhibition of the U87 MG-derived cell lines, we tested its efficacy against A431 xenografts. This squamous cell carcinoma-derived cell line contains significant EGFR gene amplification, which is retained both *in vitro* and *in vivo*. Treatment of A431 xenografts with mAb 806 produced significant growth inhibition in both a preventative and established model, indicating the antitumor effects of mAb 806 are not restricted to transfected U87 MG cell lines.

Complete prevention of A431 xenograft growth by antibody treatment has been reported previously. The wt EGFR mAbs 528, 225, and 425 all prevented the formation of A431 xenografts when administered either on the day or 1 day after tumor inoculation (9, 10). The reason for this difference in efficacy between these wt EGFR antibodies and mAb 806 is not known but may be related to the mechanism of cell growth inhibition. The wt EGFR antibodies function by blocking ligand binding to the EGFR, but this is probably not the case with mAb 806 because it only binds a small EGFR subset on the surface of A431 cells. The significant efficacy of mAb 806 against U87 MG cells expressing the ligand-independent de2-7 EGFR further supports the notion that this antibody mediates its antitumor activity by a mechanism not involving ligand blockade. Therefore, we are currently investigating the nonimmunological and immunological mechanisms that contribute to the antitumor effects of mAb 806. Nonimmunological mechanisms may include subtle changes in receptor levels, blockade of signaling, or induction of inappropriate signaling.

Previously, agents such as doxorubicin and cisplatin in conjunction with wt EGFR antibodies have produced enhanced antitumor activity (35, 36). The combination of doxorubicin and mAb 528 resulted in total eradication of established A431 xenografts, whereas treatment with either agent alone caused only temporary *in vivo* growth inhibition (36). Likewise, the combination of cisplatin and either mAb 528 or 225 also led to the eradication of well-established A431 xenografts, which was not observed when treatment with either agent was used (35). Thus, future studies involving the combination of chemotherapeutic agents with mAb 806 are planned using xenograft models.

Maybe the most important advantage of mAb 806 compared with current EGFR antibodies is that it should be possible to directly conjugate cytotoxic agents to mAb 806. This approach is not feasible with current EGFR-specific antibodies because they target the liver and cytotoxic conjugation would almost certainly induce severe toxicity. Given that mAb 806 failed to bind U87 MG cells expressing  $1 \times 10^5$  EGFRs and our initial immunohistochemical analysis showing that mAb 806 does not bind normal liver, we believe that it is unlikely this antibody will target normal liver. However, formal demonstration of this requires ongoing analysis and ultimately clinical trials in cancer patients. Conjugation of cytotoxic agents such as drugs (37) or radioisotopes (38) to antibodies has the potential to improve efficacy and reduce the systemic toxicity of these agents. Furthermore, it is likely that the direct antitumor effects of mAb 806 reported here would be further enhanced by the coupling of appropriate cytotoxics.

This study clearly demonstrates that mAb 806 has significant *in vivo* antitumor activity against de2-7 EGFR-positive xenografts and



tumors overexpressing the EGFR. The unique specificity of mAb 806 suggests immunotherapeutic potential in targeting a number of tumor types, particularly head and neck tumors and glioma, without the restrictions associated with normal tissue uptake. Finally, given that systemic administration of mAb 806 inhibits the growth of intracranial glioma xenografts,<sup>4</sup> we plan to conduct clinical trials with mAb 806 in patients with glioma.

## References

- Scott, A. M., and Welt, S. Antibody-based immunological therapies. *Curr. Opin. Immunol.*, 9: 717-722, 1997.
- Fan, Z., and Mendelsohn, J. Therapeutic application of anti-growth factor receptor antibodies. *Curr. Opin. Oncol.*, 10: 67-73, 1998.
- Garcia de Palazzo, I. E., Adams, G. P., Sundareshan, P., Wong, A. J., Testa, J. R., Bigner, D. D., and Weiner, L. M. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res.*, 53: 3217-3220, 1993.
- Wikstrand, C. J., Reist, C. J., Archer, G. E., Zalutsky, M. R., and Bigner, D. D. The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. *J. Neurovirol.*, 4: 148-158, 1998.
- Korshunov, A., Golanov, A., Sycheva, R., and Pronin, I. Prognostic value of tumour associated antigen immunoreactivity and apoptosis in cerebral glioblastomas: an analysis of 168 cases. *J. Clin. Pathol.*, 52: 574-580, 1999.
- Grandis, J. R., Melhem, M. F., Gooding, W. E., Day, R., Holst, V. A., Wagener, M. M., Drenning, S. D., and Twardy, D. J. Levels of TGF- $\alpha$  and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J. Natl. Cancer Inst.*, 90: 824-832, 1998.
- Fan, Z., Masui, H., Altas, I., and Mendelsohn, J. Blockade of epidermal growth factor receptor function by bivalent and monovalent fragments of 225 anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res.*, 53: 4322-4328, 1993.
- Teramoto, T., Onda, M., Tokunaga, A., and Asano, G. Inhibitory effect of anti-epidermal growth factor receptor antibody on a human gastric cancer. *Cancer (Phila.)*, 77: 1639-1645, 1996.
- Rodeck, U., Herlyn, M., Herlyn, D., Molthoff, C., Atkinson, B., Varelo, M., Steplewski, Z., and Koprowski, H. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects. *Cancer Res.*, 47: 3692-3696, 1987.
- Masui, H., Kawamoto, T., Sato, J. D., Wolf, B., Sato, G., and Mendelsohn, J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res.*, 44: 1002-1007, 1984.
- Faillot, T., Magdelenat, H., Mady, E., Stasiecki, P., Fohanno, D., Gropp, P., Poisson, M., and Delattre, J. Y. A Phase I study of an anti-epidermal growth factor receptor monoclonal antibody for the treatment of malignant gliomas. *Neurosurgery*, 39: 478-483, 1996.
- Divgi, C. R., Welt, S., Kris, M., Real, F. X., Yeh, S. D., Gralla, R., Merchant, B., Schweighart, S., Unger, M., Larson, S. M., et al. Phase I and imaging trial of indium 111-labeled anti-epidermal growth factor receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma. *J. Natl. Cancer Inst.*, 83: 97-104, 1991.
- Baselga, J., Pfister, D., Cooper, M. R., Cohen, R., Burtmess, B., Bos, M., D'Andrea, G., Seidman, A., Norton, L., Gunnert, K., Falcey, J., Anderson, V., Waksal, H., and Mendelsohn, J. Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. *J. Clin. Oncol.*, 18: 904, 2000.
- Voldborg, B. R., Damstrup, L., Spang-Thomsen, M., and Poulsen, H. S. Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Ann. Oncol.*, 8: 1197-1206, 1997.
- Sugawa, N., Ekstrand, A. J., James, C. D., and Collins, V. P. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc. Natl. Acad. Sci. USA*, 87: 8602-8606, 1990.
- Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., and Huang, H. J. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc. Natl. Acad. Sci. USA*, 91: 7727-7731, 1994.
- Tang, C. K., Gong, X. Q., Moscatello, D. K., Wong, A. J., and Lippman, M. E. Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer. *Cancer Res.*, 60: 3081-3087, 2000.
- Olapade-Olaopa, E. O., Moscatello, D. K., MacKay, E. H., Horsburgh, T., Sandhu, D. P., Terry, T. R., Wong, A. J., and Habib, F. K. Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. *Br. J. Cancer*, 82: 186-194, 2000.
- Moscatello, D. K., Holgado-Madruga, M., Godwin, A. K., Ramirez, G., Gunn, G., Zolnick, P. W., Biegel, J. A., Hayes, R. L., and Wong, A. J. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res.*, 55: 5536-5539, 1995.
- Wikstrand, C. J., Hale, L. P., Batra, S. K., Hill, M. L., Humphrey, P. A., Kurpad, S. N., McLendon, R. E., Moscatello, D., Pegram, C. N., Reist, C. J., et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res.*, 55: 3140-3148, 1995.
- Hills, D., Rowlinson-Busza, G., and Gullick, W. J. Specific targeting of a mutant, activated FGF receptor found in glioblastoma using a monoclonal antibody. *Int. J. Cancer*, 63: 537-543, 1995.
- Okamoto, S., Yoshikawa, K., Obata, Y., Shibuya, M., Aoki, S., Yoshida, J., and Takahashi, T. Monoclonal antibody against the fusion junction of a deletion-mutant epidermal growth factor receptor. *Br. J. Cancer*, 73: 1366-1372, 1996.
- Nagane, M., Coufal, F., Lin, H., Bogler, O., Cavenee, W. K., and Huang, H. J. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res.*, 56: 5079-5086, 1996.
- Sato, J. D., Le, A. D., and Kawamoto, T. Derivation and assay of biological effects of monoclonal antibodies to epidermal growth factor receptors. *Methods Enzymol.*, 146: 63-81, 1987.
- Huang, H. S., Nagane, M., Klingbeil, C. K., Lin, H., Nishikawa, R., Ji, X. D., Huang, C. M., Gill, G. N., Wiley, H. S., and Cavenee, W. K. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J. Biol. Chem.*, 272: 2927-2935, 1997.
- Clarke, K., Lee, F. T., Brechbiel, M. W., Smyth, F. E., Old, L. J., and Scott, A. M. Therapeutic efficacy of anti-Lewis(y) humanized 3S193 radioimmunotherapy in a breast cancer model: enhanced activity when combined with Taxol chemotherapy. *Clin. Cancer Res.*, 6: 3621-3628, 2000.
- Atlas, I., Mendelsohn, J., Baselga, J., Fair, W. R., Masui, H., and Kumar, R. Growth regulation of human renal carcinoma cells: role of transforming growth factor  $\alpha$ . *Cancer Res.*, 52: 3335-3339, 1992.
- Perez-Soler, R., Donato, N. J., Shin, D. M., Rosenblum, M. G., Zhang, H. Z., Tornos, C., Brewer, H., Chan, J. C., Lee, J. S., Hong, W. K., et al. Tumor epidermal growth factor receptor studies in patients with non-small-cell lung cancer or head and neck cancer treated with monoclonal antibody RG 83852. *J. Clin. Oncol.*, 12: 730-739, 1994.
- Wersall, P., Ohlsson, I., Biberfeld, P., Collins, V. P., von Krusenstjerna, S., Larsson, S., Mellstedt, H., and Boethius, J. Intratumoral infusion of the monoclonal antibody, mAb 425, against the epidermal-growth-factor receptor in patients with advanced malignant glioma. *Cancer Immunol. Immunother.*, 44: 157-164, 1997.
- Brady, L. W., Miyamoto, C., Woo, D. V., Rackover, M., Emrich, J., Bender, H., Dadgar, S., Steplewski, Z., Koprowski, H., Black, P., et al. Malignant astrocytomas treated with iodine-125 labeled monoclonal antibody 425 against epidermal growth factor receptor: a Phase II trial. *Int. J. Radiat. Oncol. Biol. Phys.*, 22: 225-230, 1992.
- Reist, C. J., Archer, G. E., Kurpad, S. N., Wikstrand, C. J., Vaidyanathan, G., Willingham, M. C., Moscatello, D. K., Wong, A. J., Bigner, D. D., and Zalutsky, M. R. Tumor-specific anti-epidermal growth factor receptor variant III monoclonal antibodies: use of the tyramine-cellobiose radioiodination method enhances cellular retention and uptake in tumor xenografts. *Cancer Res.*, 55: 4375-4382, 1995.
- Reist, C. J., Archer, G. E., Wikstrand, C. J., Bigner, D. D., and Zalutsky, M. R. Improved targeting of an anti-epidermal growth factor receptor variant III monoclonal antibody in tumor xenografts after labeling using *N*-succinimidyl 5-iodo-3-pyridinecarboxylate. *Cancer Res.*, 57: 1510-1515, 1997.
- Sampson, J. H., Crotty, L. E., Lee, S., Archer, G. E., Ashley, D. M., Wikstrand, C. J., Hale, L. P., Small, C., Dranoff, G., Friedman, A. H., Friedman, H. S., and Bigner, D. D. Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors. *Proc. Natl. Acad. Sci. USA*, 97: 7503-7508, 2000.
- Films, J., Trent, J. M., Pollak, M. N., and Buick, R. N. Epidermal growth factor receptor gene-amplified MDA-468 breast cancer cell line and its nonamplified variants. *Mol. Cell. Biol.*, 7: 251-257, 1987.
- Fan, Z., Baselga, J., Masui, H., and Mendelsohn, J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus *cis*-diamminedichloroplatinum on well established A431 cell xenografts. *Cancer Res.*, 53: 4637-4642, 1993.
- Baselga, J., Norton, L., Masui, H., Pandiella, A., Coplan, K., Miller, W. H., Jr., and Mendelsohn, J. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J. Natl. Cancer Inst.*, 85: 1327-1333, 1993.
- Trail, P. A., and Bianchi, A. B. Monoclonal antibody drug conjugates in the treatment of cancer. *Curr. Opin. Immunol.*, 11: 584-588, 1999.
- DeNardo, S. J., Kroger, L. A., and DeNardo, G. L. A new era for radiolabeled antibodies in cancer? *Curr. Opin. Immunol.*, 11: 563-569, 1999.

<sup>4</sup> K. Mishima, T. G. Johns, A. M. Scott, E. Stockert, X.-D. Ji, P. Suvana, J. R. Volland, L. J. Old, H.-J. S. Huang, and W. K. Cavenee. Growth suppression of intracranial xenografted glioblastomas overexpressing mutant epidermal growth factor receptors by systemic administration of mAb 806, a novel monoclonal antibody directed to the receptor; submitted for publication.